

| | | | |
|--|--|---|---|
| FORM PTO-1390 (REV 12-29-99) | | U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE | ATTORNEY'S DOCKET NUMBER 13216-73220 |
| TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371 | | | U.S. APPLICATION NO. (if known, see 37 CFR 1.5) 09/743840 |
| INTERNATIONAL APPLICATION NO. PCT/US99/16001 | INTERNATIONAL FILING DATE 15 July 1999 (15.07.99) | PRIORITY DATE CLAIMED 17 July 1998 (17.07.98) | |
| TITLE OF INVENTION AGROBACTERIUM-MEDIATED TRANSFORMATION OF TURFGRASS | | | |
| APPLICANT(S) FOR DO/EO/US ZILINSKAS, Barbara A.; PITCHER Lynne H.; and LAKKARAJU Subha | | | |

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
 - a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☒ has been transmitted by the International Bureau.
 - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
 - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ have been transmitted by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☐ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☒ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☐ A FIRST preliminary amendment.
☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information:

Copy of IB/306

09/743840

PCT/US99/16001

13216-73220

17 JAN 2001

17. ☒ The following fees are submitted:**BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :**

Neither international preliminary examination fee (37 CFR 1.482)
nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO
and International Search Report not prepared by the EPO or JPO \$970.00

International preliminary examination fee (37 CFR 1.482) not paid to
USPTO but International Search Report prepared by the EPO or JPO \$840.00

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but
international search fee (37 CFR 1.445(a)(2)) paid to USPTO \$690.00

International preliminary examination fee paid to USPTO (37 CFR 1.482)
but all claims did not satisfy provisions of PCT Article 33(1)-(4) \$670.00

International preliminary examination fee paid to USPTO (37 CFR 1.482)
and all claims satisfied provisions of PCT Article 33(1)-(4) \$96.00

ENTER APPROPRIATE BASIC FEE AMOUNT =**CALCULATIONS** PTO USE ONLY

\$ 100.00

Surcharge of \$130.00 for furnishing the oath or declaration later than ☐ 20 ☒ 30
months from the earliest claimed priority date (37 CFR 1.492(e)).

\$ 130.00

| CLAIMS | NUMBER FILED | NUMBER EXTRA | RATE |
|---|--------------|--------------|------------|
| Total claims | 21 - 20 = | 1 | X \$18.00 |
| Independent claims | 2 - 3 = | 0 | X \$78.00 |
| MULTIPLE DEPENDENT CLAIM(S) (if applicable) | | | + \$260.00 |

\$ 18.00

\$ 0.00

\$ 0.00

TOTAL OF ABOVE CALCULATIONS =

\$ 248.00

Reduction of 1/2 for filing by small entity, if applicable. A Small Entity Statement
must also be filed (Note 37 CFR 1.9, 1.27, 1.28).

\$ 124.00

SUBTOTAL =

\$ 124.00

Processing fee of \$130.00 for furnishing the English translation later than ☐ 20 ☐ 30
months from the earliest claimed priority date (37 CFR 1.492(f)).

\$

TOTAL NATIONAL FEE =

\$

Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be
accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property

\$ 40.00

TOTAL FEES ENCLOSED =

\$ 164.00

Amount to be
refunded: \$

charged: \$

a. ☒ A check in the amount of \$ 164.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of \$ _____ to cover the above fees.
A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any
overpayment to Deposit Account No. 50-1089. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO

Janet E. Reed
Saul Ewing
Centre Square West
1500 Market Street, 38th Floor
Philadelphia, PA 19102-2186
US

SIGNATURE

Janet E. Reed

NAME

36,252

REGISTRATION NUMBER

PTO/PCT Rec'd 17 JAN 2001

AGROBACTERIUM-MEDIATED TRANSFORMATION
OF TURFGRASS

This application claims priority to U. S. Provisional Application No. 60/093,163, filed July 17, 1998, the entirety of which is incorporated by reference herein.

5

FIELD OF THE INVENTION

This invention relates to the field of plant transformation methods. More specifically, an *Agrobacterium*-mediated method for transforming turfgrass is provided, as well as transgenic turfgrass produced by the method.

BACKGROUND OF THE INVENTION

Various scientific articles and patents are referred to in parentheses throughout the specification. These documents are incorporated by reference herein to describe the state of the art to which this invention pertains. Full citations of the scientific articles appear at the end of the specification.

Turfgrasses and turfgrass breeding are of significant economic importance worldwide. In recent years, traditional breeding programs have been augmented by molecular biological and recombinant techniques. However, similar to most monocotyledenous plants, turfgrasses have proven recalcitrant to tissue culture, transformation and regeneration procedures. Among these, *Agrobacterium*-mediated transformation of a turfgrass heretofore has not been accomplished.

Turfgrass transformation has been achieved using direct methods of DNA transfer, including protoplast transformation and particle gun bombardment.

- 2 -

Nonetheless, *Agrobacterium*-mediated transformation offers several advantages over particle gun bombardment or other means of direct gene transfer. These include stable transgene integration without rearrangement of either
5 host or transgene DNA; preferential integration of the transgene into transcriptionally active regions of the genome; ability to transfer large pieces of DNA; and integration of low numbers of gene copies into plant nuclear DNA which is particularly important to minimize
10 possible co-suppression of the transgene in later generations.

Until recently, *Agrobacterium*-mediated transformation was thought to be limited to dicotyledonous plants. However, Hiei et al. in 1994
15 described efficient transformation of rice by *Agrobacterium*, and subsequently there have been convincing reports for maize, barley and wheat (Ishida et al., 1996; Tingay et al., 1997; Cheng et al., 1997; see also U.S. Patent No. 5,591,616 to Hiei et al). Numerous
20 factors are of critical importance in *Agrobacterium*-mediated transformation of monocots, including the type and stage of tissue that is infected, the vector and bacterial strains used, plant genotype, tissue culture conditions, and the actual infection process. As a
25 result, methods that have proven successful for *Agrobacterium*-mediated transformation of some monocots, such as rice and maize, have not been successful for transforming turfgrass.

An object of the present invention is to
30 develop an efficient and reliable transformation system for turfgrass, mediated by *Agrobacterium tumefaciens*. Another object of the invention is to regenerate transgenic plants containing one or more foreign genes introduced by *Agrobacterium tumefaciens*-mediated
35 transformation.

- 3 -

SUMMARY OF THE INVENTION

In accordance with the objects of the present invention, an *Agrobacterium*-mediated transformation system for turfgrass is provided, which is efficient and reliable. The invention also provides for the development of transgenic plants containing one or more transgenes of significant practical utility.

According to one aspect of the invention, a method of producing a transgenic turfgrass plant is provided. The method comprises: (a) providing regenerable callus tissue from the turfgrass plant; (b) inoculating the tissue with *Agrobacterium* carrying at least one vector for transformation, the vector comprising virulence genes that confer strong infectivity to *Agrobacterium*, in which vector is inserted a heterologous DNA construct operably linked to a promoter from a monocotyledonous species, and a selectable marker gene conferring antibiotic resistance to transformed cells operably linked to a promoter from a monocotyledonous species; (c) culturing the inoculated tissue under conditions that enable the *Agrobacterium* vector to transform cells of the tissue; (d) selectively culturing the inoculated tissue on a selection medium comprising the antibiotic; and (e) regenerating a transformed turfgrass plant from the selectively cultured tissue.

Preferably, the turfgrass is a species selected from the group consisting of creeping bentgrass, tall fescue, velvet bentgrass, perennial ryegrass, hard fescue, Chewings fescue, strong creeping fescue, colonial bentgrass and Kentucky bluegrass. In another preferred embodiment, the *Agrobacterium* comprises a binary vector system and the virulence genes therein are obtained from a plasmid within *Agrobacterium tumefaciens* strain 281. The promoter is preferably selected from the group

- 4 -

consisting of maize ubiquitin gene promoters, rice actin gene promoters, maize *Adh 1* gene promoters, rice or maize tubulin (*Tub A*, *B* or *C*) gene promoters, and alfalfa *His 3* gene promoters. The selectable marker gene preferably
5 confers hygromycin resistance on transformed tissue. The callus used for the transformation preferably is obtained by culturing seeds of the turfgrass on a de-differentiation medium.

Also provided in accordance with another aspect
10 of the invention is a transgenic turfgrass plant prepared by the aforementioned method. Seeds of the transgenic plant are provided as well. In preferred embodiments, the transgenic turfgrass plant comprises a transgene selected from the group consisting of genes encoding
15 glucose oxidase, citrate synthase, Δ -9 desaturase from *Saccharomyces cerevisiae* or *Cryptococcus curvatus*, Δ -11 desaturase, a plant homolog of the neutrophil NADPH oxidase, a bacteriopsin from *Halobacterium halobium*, or pokeweed antiviral protein.

According to another aspect of the invention, a
20 superbinary vector for *Agrobacterium*-mediated transformation of turfgrass is provided. The vector comprises: (a) a virulence region from a Ti plasmid of an *A. tumefaciens* strain that confers to the strain as
25 strong a virulence as that displayed by *A. tumefaciens* strain 281; (b) a selectable marker gene operably linked to a promoter obtained from a gene of a monocotyledenous plant; and (c) a site for insertion of at least one additional coding sequence, operably linked to a promoter
30 obtained from a gene of a monocotyledenous plant, the promoter being the same as or different from the promoter operably linked to the selectable marker gene. In preferred embodiments the virulence region is obtained from *Agrobacterium* strain 281. The promoter is selected

- 5 -

from the group consisting of maize ubiquitin gene promoters, rice actin gene promoters, maize *Adh* 1 gene promoters, rice or maize tubulin (*Tub* A, B or C) gene promoters, and alfalfa *His* 3 gene promoters. The
5 selectable marker gene preferably confers hygromycin resistance on transformed cells. The site for insertion of the additional coding sequence preferably comprises a coding sequence of a reporter gene, and/or comprises a coding sequence of a gene encoding the useful proteins
10 listed above.

According to another aspect of the invention, a turfgrass plant cell transformed with the aforementioned *Agrobacterium* vector is provided. Preferably the turfgrass is creeping bentgrass, tall fescue, velvet
15 bentgrass, perennial ryegrass, hard fescue, Chewings fescue, strong creeping fescue, colonial bentgrass or Kentucky bluegrass. A transgenic turfgrass plant regenerated from the aforementioned transformed cell is also provided, as are seeds of the transgenic turfgrass
20 plant.

Other features and advantages of the present invention will be understood by reference to the detailed description and examples that follow.

25

DETAILED DESCRIPTION OF THE INVENTION

I. Definitions

Various terms used throughout the specification and claims to describe the invention. Unless otherwise
30 specified, these terms are defined as set forth below.

With reference to nucleic acid molecules, the term "isolated nucleic acid" is sometimes used. This term, when applied to DNA, refers to a DNA molecule that is separated from sequences with which it is immediately

- 6 -

contiguous (in the 5' and 3' directions) in the naturally occurring genome of the organism from which it was derived. For example, the "isolated nucleic acid" may comprise a DNA molecule inserted into a vector, such as a
5 plasmid or virus vector, or integrated into the genomic DNA of a prokaryote or eukaryote. An "isolated nucleic acid molecule" may also comprise a cDNA molecule.

With respect to RNA molecules, the term "isolated nucleic acid" primarily refers to an RNA
10 molecule encoded by an isolated DNA molecule as defined above. Alternatively, the term may refer to an RNA molecule that has been sufficiently separated from RNA molecules with which it would be associated in its natural state (i.e., in cells or tissues), such that it
15 exists in a "substantially pure" form (the term "substantially pure" is defined below).

The term "substantially pure" refers to a preparation comprising at least 50-60% by weight the compound of interest (e.g., nucleic acid,
20 oligonucleotide, protein, etc.). More preferably, the preparation comprises at least 75% by weight, and most preferably 90-99% by weight, the compound of interest. Purity is measured by methods appropriate for the compound of interest (e.g. chromatographic methods,
25 agarose or polyacrylamide gel electrophoresis, HPLC analysis, and the like).

When used herein in describing components of media or other experimental results, the term "about" means within a margin of commonly acceptable error for
30 the determination being made, using standard methods. For tissue culture media in particular, persons skilled in the art would appreciate that the concentrations of various components initially added to culture media may

- 7 -

change somewhat during use of the media, e.g., by evaporation of liquid from the medium or by condensation onto the medium. Moreover, it is understood that the precise concentrations of the macronutrients, vitamins
5 and carbon sources are less critical to the efficacy of the media than are the micronutrient, hormone and antibiotic concentrations.

Nucleic acid sequences and amino acid sequences can be compared using computer programs that align the
10 similar sequences of the nucleic or amino acids thus define the differences. In the comparisons made in the present invention, the CLUSTLW program and parameters employed therein were utilized (Thompson et al., 1994, *supra*). However, equivalent alignments and
15 similarity/identity assessments can be obtained through the use of any standard alignment software. For instance, the BLAST programs used to query sequence similarity in GenBank and other public databases may be used. The GCG Wisconsin Package version 9.1, available
20 from the Genetics Computer Group in Madison, Wisconsin, and the default parameters used (gap creation penalty=12, gap extension penalty=4) by that program may also be used to compare sequence identity and similarity.

The term "substantially the same" refers to
25 nucleic acid or amino acid sequences having sequence variations that do not materially affect the functionality of *cis* acting regulatory sequences (e.g., promoters, transcriptional response elements, etc.) or the nature of the encoded gene product (i.e. the
30 structure, stability characteristics, substrate specificity and/or biological activity of the protein). With particular reference to nucleic acid sequences, the term "substantially the same" is intended to refer to

- 8 -

conserved sequences governing expression and to the coding region (referring primarily to degenerate codons encoding the same amino acid, or alternate codons encoding conservative substitute amino acids in the encoded polypeptide).

The terms "percent identical" and "percent similar" are also used herein in comparisons among nucleic acid sequences. When referring to nucleic acid molecules, "percent identical" refers to the percent of the nucleotides of the subject nucleic acid sequence that have been matched to identical nucleotides by a sequence analysis program. When referring to amino acid sequences, "percent identical" refers to the percent of the amino acids of the subject amino acid sequence that have been matched to identical amino acids in the compared amino acid sequence by a sequence analysis program. "Percent similar" refers to the percent of the amino acids of the subject amino acid sequence that have been matched to identical or conserved amino acids. Conserved amino acids are those which differ in structure but are similar in physical properties such that the exchange of one for another would not appreciably change the tertiary structure of the resulting protein. Conservative substitutions are defined in Taylor (1986, J. Theor. Biol. 119:205).

With respect to oligonucleotides or other single-stranded nucleic acid molecules, the term "specifically hybridizing" refers to the association between two single-stranded nucleic acid molecules of sufficiently complementary sequence to permit such hybridization under pre-determined conditions generally used in the art (sometimes termed "substantially complementary"). In particular, the term refers to

- 9 -

hybridization of an oligonucleotide with a substantially complementary sequence contained within a single-stranded DNA or RNA molecule, to the substantial exclusion of hybridization of the oligonucleotide with single-stranded nucleic acids of non-complementary sequence.

A "coding sequence" or "coding region" refers to a nucleic acid molecule having sequence information necessary to produce a gene product, when the sequence is expressed.

The term "operably linked" or "operably inserted" means that the regulatory sequences necessary for expression of the coding sequence are placed in a nucleic acid molecule in the appropriate positions relative to the coding sequence so as to enable expression of the coding sequence. This same definition is sometimes applied to the arrangement other transcription control elements (e.g. enhancers) in an expression vector.

When describing the organization of a nucleic acid molecule, the term "upstream" refers to the 5' direction and the term "downstream" refers to the 3' direction.

The term "reporter gene" refers to a nucleic acid coding sequence that encodes a readily detectable gene product, which may be operably linked to a promoter region to form a chimeric gene, such that expression of the coding sequence is regulated by the promoter and the product of the coding sequence is readily assayed.

The term "selectable marker gene" refers to a gene that when expressed confers a selectable phenotype, such as antibiotic resistance, on a transformed cell or plant.

Transcriptional and translational control sequences, sometimes referred to herein as "expression

- 10 -

control" sequences or elements, or "expression
regulating" sequences or elements, are DNA regulatory
elements such as promoters, enhancers, ribosome binding
sites, polyadenylation signals, terminators, and the
5 like, that provide for the expression of a coding
sequence in a host cell. The term "expression" is
intended to include transcription of DNA and translation
of the mRNA transcript.

The terms "promoter", "promoter region" or
10 "promoter sequence" refer generally to transcriptional
regulatory regions of a gene, which may be found at the
5' or 3' side of the coding region, or within the coding
region, or within introns. Typically, a promoter is a
DNA regulatory region capable of binding RNA polymerase
15 in a cell and initiating transcription of a downstream
(3' direction) coding sequence. The typical 5' promoter
sequence is bounded at its 3' terminus by the
transcription initiation site and extends upstream (5'
direction) to include the minimum number of bases or
20 elements necessary to initiate transcription at levels
detectable above background. Within the promoter
sequence is a transcription initiation site (conveniently
defined by mapping with nuclease S1), as well as protein
binding domains (consensus sequences) responsible for the
25 binding of RNA polymerase.

A "vector" is a replicon, such as plasmid,
phage, cosmid, or virus to which another nucleic acid
segment may be operably inserted so as to bring about the
replication or expression of the segment.

30 The term "nucleic acid construct" or "DNA
construct" refers to genetic sequence used to transform
plant cells and generate progeny transgenic plants. At
minimum a DNA construct comprises a coding region for a

- 11 -

selected gene product, operably linked to 5' and 3' regulatory sequences for expression in transformed plants. In preferred embodiments, such constructs are chimeric, i.e., the coding sequence is from a different source one or more of the regulatory sequences (e.g., coding sequence from tobacco and promoter from maize). However, non-chimeric DNA constructs also can be used. In addition to methods specifically described herein, the transforming DNA may be prepared according to standard protocols such as those set forth in Ausubel et al. (1999). A plant species or cultivar may be transformed with a DNA construct (chimeric or non-chimeric) that encodes a polypeptide from a different plant species or cultivar, or a non-plant species. Alternatively, a plant species or cultivar may be transformed with a DNA construct (chimeric or non-chimeric) that encodes a polypeptide from the same plant species or cultivar. The term "transgene" is sometimes used to refer to the DNA construct within the transformed cell or plant.

20 A cell has been "transformed" or "transfected" by a DNA construct when such DNA has been introduced inside the cell. The transforming DNA may or may not be integrated (covalently linked) into the genome of the cell. For example, the transforming DNA may be maintained on an episomal element such as a plasmid. With respect to eukaryotic cells, a stably transformed cell is one in which the transforming DNA has become integrated into a chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eukaryotic cell to establish cell lines or clones comprised of a population of daughter cells containing the transforming DNA. A "clone" is a population of cells derived from a single cell or common ancestor by mitosis.

- 12 -

A "cell line" is a clone of a primary cell that is capable of stable growth *in vitro* for many generations.

II. Description

5 The present invention provides an efficient and reliable transformation system for turfgrass species. The species chosen to develop this system uses creeping bentgrass, *Agrostis palustris* Huds. Due to the reasonably close similarity among various turfgrasses
10 (i.e., in physiology, genome organization, etc.), this transformation system will have broad applicability to many different kinds of turfgrass. For instance, the system also has been applied to velvet bentgrass, *Agrostis canina* L. and tall fescue, *Festuca arundinacea*
15 Scheb., and although the transformation efficiency is not as great as for creeping bentgrass, it is clear that transformants can be obtained from several species. Other turfgrasses contemplated for the transformation system of the invention include, but are not limited to,
20 perennial ryegrass, hard fescue, Chewings fescue, strong creeping fescue, colonial bentgrass and Kentucky bluegrass.

Binary vectors are commonly used in *Agrobacterium*-mediated transformation. Recently, a
25 "superbinary" vector system was developed (Komari et al., 1990; Saito et al., 1992; Hiei et al., 1994; U.S. Patent No. 5,731,139 to Komari et al.). In this system the plasmid that carries the T-DNA also contains certain virulence genes from strain A281, which is known for high
30 efficiency of transformation.

In arriving at the present invention, the conditions and vectors described for transformation of rice (Hiei et al., 1994) and maize (Ishida et al., 1996) were initially used, but without success in creeping
35 bentgrass. It was suspected that a major problem with

- 13 -

these vectors was the CaMV35S promoter which drives the expression of the selectable marker in the rice and maize systems. Therefore, a new vector was constructed, which has the components of the "superbinary vector" developed by Hiei et al. (1994), coupled with a suitable selectable marker (hygromycin resistance) and the easily scorable β -glucuronidase (GUS) reporter gene. More significantly, in the new vector, the expression of each of these respective transgenes is driven by promoters for strong, constitutive expression in monocots, e.g., the rice actin and maize ubiquitin promoters. Independent transformations, conducted over the course of several months, demonstrated that an *Agrobacterium tumefaciens* such as this can indeed transfer DNA into the chromosomes of various turfgrasses.

Certain features of the *Agrobacterium*-mediated turfgrass transformation system describe herein are believed to be particularly important for successful transformation and regeneration of transgenic turfgrass. One of these is the use of Ti plasmids from strongly infective *Agrobacterium* strains. Preferred for use are superbinary hybrid *Agrobacterium* vectors, such as pSB1 and pSB11 (Komari et al., 1996). These and other superbinary vectors that can be modified for use in the present invention are described in U.S. Patent No. 5,731,179 to Komari et al. These vectors contain a DNA region containing *virB* gene and *virG* gene in virulence region of Ti plasmid pTiBo542 of *Agrobacterium tumefaciens*, which is a Ti plasmid contained in *A. tumefaciens* strain A281 (ATCC Accession No. 37349), a strain known for its strong virulence. The *virB* and *virG* genes in the virulence region of pTiBo542 are contained a 15.2 kb KpnI restriction fragment, which itself may be used in the present invention. Moreover, although vectors comprising the hypervirulence-conferring portions

- 14 -

of pTiBo542 are exemplified herein, it will be appreciated by persons skilled in the art that corresponding genes from any highly virulent *Agrobacterium* strain may be used instead. These include
5 strains that are presently available as well as strains that may be discovered in the future.

A particularly important feature of the invention is the modification of *Agrobacterium* vectors to contain promoters and other regulatory sequences
10 particularly suited for expression in turfgrasses. Thus, the strong constitutive promoters from the rice actin gene and the maize ubiquitin gene are exemplified herein. However, any constitutively expressed or inducible promoter for expression in monocots may be used in the
15 present invention. Examples of other constitutive promoters suitable for use in the present invention are the maize *Adh 1* promoter, the rice or maize tubulin (*Tub A*, *B* or *C*) promoters, and the alfalfa *His 3* promoter. Monocotyledenous tissue-specific promoters also may be
20 utilized. For example, seed-specific promoters suitable for use in the present invention include zein promoters, such as the 27-kDa zein promoter or the 10-kDa zein promoter.

The choice of selectable marker is also
25 important. Hygromycin is exemplified herein as a selectable marker. However, other selectable markers are also suitable for use in the invention. For instance, resistance to phosphothricin herbicides is particularly useful for selecting transformed monocotyledenous plant
30 cells. In addition, kanamycin is routinely used as a selection medium for plant transformation, though it is less preferred for use in the present invention than is hygromycin.

For *Agrobacterium*-mediated transformation, an
35 antibiotic to eliminate *Agrobacterium* is included in the

- 15 -

selection medium. Cefotaxime is the preferred antibiotic for this purpose. However, Augmentin (amoxicillin and lithium clavulenate) and carbenicillin also have been found effective for eliminating *Agrobacterium*.

5 If a reporter gene is utilized, it can be any one of several commonly used in the art. Examples of suitable reporter genes include, but are not limited to, genes encoding β -glucuronidase (GUS), luciferase, chloramphenicol acetyl transferase (CAT), green
10 fluorescent protein (GFP) and modified forms of GFP (e.g., EGFP, EPFP and GFP_{uv}).

 In addition, the use of friable, regenerable callus for the transformation protocol is considered particularly important to successful, efficient
15 transformation and regeneration of intact plants. In a preferred embodiment, the callus is generated from embryogenic tissue, most preferably mature seeds, though it may also be generated from seed parts or immature seeds. Other organogenic tissues may also be utilized as
20 starting material for callus growth. It has been found in accordance with the present invention that the culture media described herein will facilitate the growth of appropriate (i.e. friable, regenerable) callus tissue, which is best harvested as soon as a sufficient amount of
25 callus is generated from the starting material.

 To optimize the conditions for efficient transformation of turfgrass, a number of parameters may be systematically altered to determine the best conditions to achieve efficient and easily reproducible
30 transformation. Conditions that may be altered include: 1) the culture medium and induction agents immediately prior to and during co-cultivation with *Agrobacterium*; 2) inoculation and co-culture methods and time period; 3) the presence and absence of surfactants in the

- 16 -

inoculation medium; and 4) use of embryogenic callus versus suspension cell cultures. Optimum conditions have been established for creeping bentgrass. Modifications of these conditions have enabled the transformation of callus obtained from velvet bentgrass and tall fescue.

Thus, the present invention provides a method for generating transgenic turfgrass using *Agrobacterium* vectors, that heretofore has been unavailable. A preferred embodiment of the present invention comprises the following transformation/regeneration protocol, based on *Agrobacterium*-mediated transformation.

1) The starting tissue (e.g., mature seeds in a particularly preferred embodiment) of the selected turfgrass are surface-sterilized and placed on a standard de-differentiation tissue culture medium (see the examples) for about 3-6 weeks, preferably in the dark, at room temperature.

2) Proliferating calli are selected and transferred to fresh medium of the same type on a regular basis. Only callus that is friable and regenerable should be selected for further culture.

3) Prior to exposure to *Agrobacterium*, the chosen callus is transferred to new medium to promote active cell division; the callus is used for transformation within about a week of the final transfer to fresh medium.

4) The *Agrobacterium* strain carrying the transforming plasmids is grown, induced with acetosyringone (a phenolic compound demonstrated to induce *Agrobacterium vir* gene expression) and resuspended in an inoculation medium comprising acetosyringone and, optionally, a surfactant such as pluronic F-68 or another suitable surfactant.

5) Optionally, callus may be pre-treated prior to inoculation with *Agrobacterium* by vacuum-infiltration

- 17 -

with inoculation medium containing acetosyringone.

6) The callus tissue is then placed in the presence of the *Agrobacterium* suspension to allow the bacteria to infiltrate the callus tissue. Optionally, excess *Agrobacterium* may thereafter be removed from the calli by gentle vacuum filtration. Calli, on a sterile filter, are then placed on co-cultivation solid medium (standard de-differentiation medium but containing glucose and acetosyringone). Co-cultivation of the calli with the *Agrobacterium* cells that remain associated with the calli is allowed to proceed for a few (e.g., three) days, the object being to allow sufficient time for penetration of the T-DNA into the plant cells while avoiding overgrowth of the calli with the *Agrobacterium*.

7) The calli are then transferred to selection medium containing the selection antibiotic (e.g., hygromycin) and the antibiotic for removal of the *Agrobacterium*. Calli are kept on this medium for several weeks (e.g. 6-8 weeks) and checked periodically for proliferation of the calli on the selection medium.

8) New growth that appears on calli on the selection medium is first transferred to fresh selection medium and allowed to proliferate. This first selection is best performed when the new growth is as small as possible in order to ensure that independent transformants are selected and proliferated in the absence of other independent transformants.

9) After sufficient proliferation, a small portion of each of the putatively transformed calli is tested for expression of the gene of interest, and/or for expression of a detectable marker gene (e.g., GUS activity). The remaining portions of positive-testing calli are retained on the selection medium for continued subculture and proliferation. Subsequently, the transformed calli are transferred to a regeneration

- 18 -

medium containing growth regulators to promote shoot differentiation.

10) When sufficiently developed, the shoots are transferred to a second regeneration medium formulated to further stimulate root growth. After a sufficient growth phase, plantlets are transferred to either new tissue culture medium or to soil or equivalent planting media.

This invention provides transgenic turfgrass produced by the above-described methods, and also is intended to encompass cells and tissues of those plants, including, but not limited to, leaves, stems, shoots, roots, flowers, fruits and seeds. In a preferred embodiment, seeds of the transgenic plants produced by the methods of the invention are provided.

The plants grown from the aforementioned seeds, or seeds from other turfgrass species or varieties, or the progeny thereof, all of which are considered within the scope of this invention, are used in crosses and selection methods to transfer genes of interest into other turfgrass genotypes, cultivars, varieties and the like.

Plants grown from transgenic turfgrass seeds can also be used to detect the presence of the inserted transgene and vector sequences using DNA extraction, cleavage by one or more restriction endonucleases, and analysis, e.g., Southern blotting using probes derived from the gene or genes of interest. In this manner, the transfer of foreign genes into progeny of breeding crosses can be monitored.

The *Agrobacterium*-mediated turfgrass transformation system of the present invention can be used to introduce many genes of interest into different turfgrass species or varieties. Accordingly, the present invention provides several specific hybrid vectors for *Agrobacterium*-mediated transformation of turfgrass. A

- 19 -

number of gene constructs are of considerable practical utility as used to create one or more different transgenic turfgrasses. These include: 1) the gene encoding glucose oxidase from *Aspergillus niger* which when expressed in potato provides resistance to bacterial and fungal pathogens through its production of H₂O₂ in the plant apoplast (Wu et al., 1995); 2) the gene encoding citrate synthase from *Pseudomonas aeruginosa* which when expressed in the cytoplasm of tobacco provides tolerance to toxic levels of aluminum in the soil (Manuel et al., 1997); 3) the genes encoding Δ -9 desaturase from *Saccharomyces cerevisiae* (Stukey et al., 1990) and *Cryptococcus curvatus* (Meesters et al., 1997) and Δ -11 desaturase from *Trichoplosia ni* (Knipple et al., 1998). When the *ole1* gene from yeast was expressed in tomato and eggplant, it provided resistance to fungal pathogens (Wang et al., 1998); 4) the recently identified gene encoding a plant homolog of the neutrophil NADPH oxidase which is thought to be responsible for the oxidative burst which is critical in plant defense against pathogens (Keller et al., 1998); 5) the gene encoding bacteriopsin, a proton pump from the bacterium, *Halobacterium halobium*, which has been demonstrated to protect transgenic tobacco expressing the protein against plant pathogens (Mittler et al., 1995); and 6) the gene encoding pokeweed antiviral protein, which is a ribosome-inactivating protein from the plant *Phytolacca americana*; expression of this gene in tobacco provides resistance to viral and fungal pathogens (Lodge et al., 1993; Zoubenko et al., 1997).

The following examples are provided to describe the invention in greater detail. They are intended to illustrate, not to limit, the invention.

- 20 -

EXAMPLE 1

Construction of *Agrobacterium tumefaciens* Strain LBA4404, containing pSB111SH Superbinary Hybrid Vector

5 The vector pTOK233 described by Hiei et al. (1994) for transformation of rice was not useful for transformation of creeping bentgrass despite numerous attempts. It was suspected that the cauliflower mosaic 35S promoter was the source of the problem in that it appears to be a very weak promoter in turfgrass. Accordingly, an alternate pair of plasmid vectors was obtained, namely pSB1 and pSB11 (Japan Tobacco, Inc. Plant Breeding and Genetics Research Laboratory, 700 Higashibara, Toyoda, Iwata, Shizuoka, Japan). pSB11 is a 15 6.3 kb plasmid which contains a multicloning region with a number of unique restriction sites between the left and right border sequences delineating the T-DNA region that will be transferred from *Agrobacterium tumefaciens* to the plant genome. Monocot "expression cassettes" expressing 20 hygromycin B phosphotransferase (to confer hygromycin resistance) and β -glucuronidase (GUS, as a scorable reporter) were inserted into pSB11 into the multiple cloning region between the T-DNA border sequences. The following strategy was used.

25 First, plasmid pACH1 (provided by Dr. German Spangenberg) was partially digested with Sac I which yielded several restriction fragments, of which the 2645 bp fragment was gel-purified. This fragment contains a 3' modified *hph* coding sequence (conferring hygromycin 30 resistance) cloned inframe and downstream from the Act 1 5' promoter sequence, first exon (non-coding), first intron and a portion of the second, ATG-containing exon of the gene. Transcription termination signals are provided by the CaMV35S terminator. This 2645 bp 35 fragment was cloned into the unique Sac I restriction site between the right and left border sequences of

- 21 -

plasmid pSB11. This plasmid was named pSB11S. Next, a 4175 bp *Hind* III fragment was excised from plasmid pAHC25, kindly provided by Dr. Peter Quail. This fragment contains the promoter, 5' untranslated exon, and first intron of the maize ubiquitin (*Ubi1*) gene fused with the GUS reporter gene coding sequence (derived from pBI101.2). This *Hind* III restriction fragment was cloned into the unique *Hind* III site of pSB11S, between the right and left border sequences of the plasmid. The resultant plasmid was named pSB11SH.

Next, this intermediate vector (pSB11SH) was electroporated into *Agrobacterium tumefaciens* LBA4404 containing the "acceptor vector" pSB1. Plasmid pSB1 is a "superbinary vector" which carries a 15.2 kb *Kpn* I fragment containing additional copies of the *vir* B, *vir* C and *vir* G virulence genes to enable efficient T-DNA transfer to monocots. Following homologous recombination between the "acceptor vector" and the "intermediate vector", a co-integrate "hybrid vector" was obtained. Bacteria expressing the drug resistance markers derived from both the acceptor and intermediate vectors were selected on AB plates containing 10 μ g/ml tetracycline and 50 μ g/ml spectinomycin, and the resultant strains carrying the "hybrid vector" were used in plant transformations.

EXAMPLE 2

30 Agrobacterium-Mediated Transformation of Creeping Bentgrass With Superbinary Hybrid Vector pSB11SH

In this example, an *Agrobacterium* transformation system for creeping bentgrass, *Agrostis palustris*, is described, which utilizes the superbinary hybrid test vector described in Example 1.

- 22 -

Media:**MMSG Medium****Per Liter:**

| | | |
|----|---|--------|
| 5 | Murashige and Skoog basal salts | 4.33 g |
| | sucrose | 30 g |
| | Gamborg vitamins (1000x) | 1 ml |
| | casein hydrolysate | 500 mg |
| | dicamba (dichloro-o-anisic acid) | 6.6 mg |
| 10 | 6 BAP (6-benzylaminopurine) | 0.5 mg |
| | Adjust the pH to 5.6 to 5.8. | |
| | Add GEL-GRO™* | 2.4 g |
| | Autoclave and dispense into Petri dishes. | |
| | (*GEL-GRO™ is gellan gum, an agar substitute, | |
| 15 | manufactured by ICN Biomedicals, Inc. If GEL-GRO™ is | |
| | unavailable, agar or an equivalent agar substitute may be | |
| | utilized.) | |

Modified AAM Medium

| | | |
|----|--|---------|
| 20 | Per Liter: | |
| | glutamine | 875 mg |
| | aspartic acid | 266 mg |
| | arginine | 174 mg |
| | glycine | 7.5 mg |
| 25 | sucrose | 88.5 mg |
| | 2,4-D | 1 mg |
| | kinetin | 0.2 mg |
| | GA ₃ | 0.1 mg |
| | Murashige & Skoog vitamins (1000x) | 1 ml |
| 30 | casamino acids | 500 mg |
| | glucose | 36 g |
| | Adjust pH to 5.2 and autoclave. | |
| | Add acetosyringone to a final concentration of 100 μM. | |

- 23 -

Regeneration Medium MSO I**Per Liter:**

| | | |
|----|-------------------------------------|--------|
| | Murashige & Skoog basal salts | 4.33 g |
| | Gamborg vitamins (1000x) | 1 ml |
| 5 | sucrose | 30 g |
| | myo-inositol | 100 mg |
| | 6-benzylaminopurine | 1 mg |
| | Adjust pH to 5.8. | |
| | Add GEL-GRO™ | 2.4 g |
| 10 | Autoclave and dispense into plates. | |

Regeneration Medium MSO II**Per Liter:**

| | | |
|----|---|--------|
| | Murashige & Skoog basal salts | 4.33 g |
| 15 | Gamborg vitamins (1000x) | 1 ml |
| | sucrose | 30 g |
| | myo-inositol | 100 mg |
| | Adjust pH to 5.8. | |
| | Add GEL-GRO™ | 2.4 g |
| 20 | Autoclave and dispense into petri dishes and plantcons. | |

AB medium

1. Prepare a stock of AB Buffer at 20X and autoclave:

| | | |
|----|----------------------------------|------|
| 25 | Per Liter: | |
| | K ₂ HPO ₄ | 60 g |
| | NaH ₂ PO ₄ | 20 g |

2. Prepare a stock of AB Salts at 20X and autoclave:

| | | |
|----|--------------------------------------|-------|
| 30 | Per Liter: | |
| | NH ₄ Cl | 20 g |
| | MgSO ₄ ·7H ₂ O | 6 g |
| | KCl | 3 g |
| | CaCl ₂ | 0.2 g |
| 35 | FeSO ₄ ·7H ₂ O | 50 mg |

- 24 -

3. For 1 liter of medium for plates:
Mix 5 g of glucose, 15 g of agar, and 900 ml of
H₂O and autoclave.
Add 50 ml of the 20X stock of AB Buffer and 50
5 ml of the 20X stock of AB Salts.
Add 10 mg tetracycline and 50 mg spectinomycin.
Pour into petri dishes.

- Production of regenerable callus:** Mature seeds
10 of creeping bentgrass (Crenshaw cultivar) were surface-
sterilized and plated on MMSG medium. The plates were
kept in the dark at room temperature for 3-6 weeks. The
proliferating calli were selected and transferred to new
MMSG medium on a regular basis. Only callus that was
15 friable and regenerable was used for transformation. The
chosen callus was transferred to new MMSG medium prior to
co-cultivation to promote active cell division and was
used for transformation within a week after transferring
to new plates. The nature of the callus (i.e.,
20 friability, regenerability and active growth) is believed
to play a key role in obtaining efficient transformation.

- Induction of *Agrobacterium tumefaciens* with
acetosyringone:** *Agrobacterium tumefaciens* LBA4404,
harboring vector pSB111SH, was streaked from a glycerol
25 stock stored at -80°C and grown at 28°C on plates
containing AB medium, supplemented with 10 µg/ml
tetracycline and 50 µg/ml spectinomycin. After three to
six days, the cells were scraped from the plate and
suspended in modified AAM medium containing 100 µM
30 acetosyringone to an OD₆₆₀ of approximately 0.5. The
bacterial suspension was left at 25°C in the dark with
shaking for 3.5 hours before using it for co-cultivation.

Co-cultivation: A given amount of friable
callus was mixed with the pre-induced *Agrobacterium*

- 25 -

suspension and incubated at room temperature in the dark for 1.5 hours. Then the contents were poured into a sterile Buchner-funnel containing a sterile Whatman filter paper. Mild vacuum was applied to drain the
5 excess *Agrobacterium* suspension. Then, the filter was moved to a plate containing MMSG medium supplemented with 100 μ M acetosyringone, and the plate was stored in the dark at room temperature for three days.

Selection and regeneration of transformants:

10 Subsequent to the three day co-cultivation, the co-cultivated calli were rinsed with 250 μ g/ml cefotaxime to suppress bacterial growth, and the calli were placed on agar plates containing MMSG medium containing 200 μ g/ml hygromycin and 250 μ g/ml cefotaxime. The calli were kept
15 in the dark at room temperature for 6-8 weeks and checked periodically for proliferation of the calli on hygromycin.

Subsequently, the hygromycin-resistant calli were placed on regeneration medium containing hygromycin and cefotaxime. The proliferating calli were first moved
20 to Regeneration Medium I (MSO I) containing cefotaxime and hygromycin. These calli were kept in the dark at room temperature for a week and were then moved to light for approximately two weeks. The tiny plants were
25 separated and transferred to deep petri plates containing Regeneration Medium II (MSO II) to promote root growth. Hygromycin and cefotaxime were included in the medium to respectively maintain selection pressure and kill any
30 remaining *Agrobacterium* cells. After 2-3 weeks, or when the plants were 1.5-2 cm tall, they were moved to plantcons containing MSO II without antibiotics. When the plants were ~10 cm tall and had developed extensive root systems, they were transferred to soil and grown in the laboratory for 3-4 weeks with 12 hours light/day.
35 The plants were transferred to 6" pots to the greenhouse,

- 26 -

where the temperature was maintained between 21° - 25°C. Supplemental lighting added approximately 50 $\mu\text{E m}^{-2} \text{ sec}^{-1}$ at canopy level when natural light was low and provided a minimal light period of 14 hours.

5 In connection with the transformation of creeping bentgrass, it should be noted that the media and protocols described below in Examples 3 and 4 may also be used to successfully transform and regenerate creeping bentgrass.

EXAMPLE 3

Agrobacterium-Mediated Transformation of Tall Fescue With Superbinary Hybrid Vector pSB111SH

15 In this example, an *Agrobacterium* transformation system for tall fescue, *Festuca arundinacea* Scheb., is described, which utilizes the superbinary hybrid test vector described in Example 1.

Media:

MMSG Medium

20 This medium was formulated as described in Example 2.

Inoculation Medium

Per Liter:

| | | |
|----|-------------------------------|--------|
| 25 | Murashige & Skoog basal salts | 440 mg |
| | sucrose | 30 g |
| | dicamba | 6.6 mg |
| | 6 BAP (6-benzyl aminopurine) | 0.5mg |
| | Gamborg vitamins (1000x) | 1ml |
| 30 | casein hydrolysate | 500 mg |
| | glucose | 10 g |

Adjust pH to 5.7 and autoclave.

Immediately before use add acetosyringone to a final concentration of 200 μM and pluronic F-68 to a final
35 concentration of 0.02%.

- 27 -

Co-Cultivation Medium**Per Liter:**

| | | |
|----|----------------------------------|---------|
| | Murashige and Skoog basal salts | 4.4 g |
| | sucrose | 30 g |
| 5 | glucose | 10 g |
| | Gamborg vitamins (1000x) | 1 ml |
| | casein hydrolysate | 500 mg |
| | dicamba (dichloro-o-anisic acid) | 6.6 mg |
| | 6 BAP (6-benzylaminopurine) | 0.5 mg |
| 10 | Adjust the pH to 5.7 | |
| | Add GEL-GRO™ | 2.4 g |
| | Autoclave | |
| | Add acetosyringone | 39.2 mg |

15 Regeneration Medium MSO I

This medium was formulated and prepared as described in Example 2.

Regeneration Medium MSO II

20 This medium was formulated and prepared as described in Example 2.

AB medium

This medium was formulated and prepared as described in Example 2.

25 **Production of regenerable callus:** Mature seeds of tall fescue were surface-sterilized and plated on MMSG medium. The plates were kept in the dark at room temperature for 3-6 weeks. The proliferating calli were selected and transferred to new MMSG medium on a regular basis. Callus chosen for transformation was transferred to new MMSG medium prior to co-cultivation to promote active cell division. The nature of the callus (i.e., friability, regenerability and active growth) is believed to play a key role in obtaining efficient transformation.

30

- 28 -

Preparation of *Agrobacterium tumefaciens*

suspension: *Agrobacterium tumefaciens* LBA4404, harboring vector pSB111SH, was streaked from a glycerol stock stored at -80°C and grown at 28°C on plates containing AB medium, supplemented with 10 µg/ml tetracycline and 50 µg/ml spectinomycin. After three to six days, the cells were scraped from the plate and suspended in inoculation medium containing 200 µM acetosyringone and 0.02% pluronic F-68 to an OD₆₆₀ between 0.5 and 0.8.

Co-cultivation of callus with *Agrobacterium*:

The friable callus chosen for transformation was placed in a sterile tube and mixed with 30 ml of *Agrobacterium* suspension. The tube was capped and covered with aluminum foil, and the contents were mixed by inversion and gently shaken for about 1.5 hr. The contents of the tube were poured into a Buchner funnel, fitted with Whatman filter. Mild vacuum was applied to flask. The filter disks containing callus with *Agrobacterium* were moved to co-cultivation plates containing acetosyringone (200 µM) and glucose at 10 g/l. The plates were sealed with parafilm and placed in the dark at room temperature for three days.

Selection and regeneration of transformants:

Subsequent to the three day co-cultivation, the co-cultivated calli were rinsed with 250 µg/ml cefotaxime solution to suppress bacterial growth, and the calli were placed on MMSG medium containing 200 µg/ml hygromycin and 250 µg/ml cefotaxime. The calli were kept in the dark at room temperature for 6-8 weeks and checked periodically for proliferation of the calli on hygromycin. The hygromycin-resistant calli were moved to new MMSG plates with hygromycin and kept in the dark at room temperature until well proliferated. Then a portion of the hygromycin-resistant callus was tested for GUS activity

- 29 -

to ensure that transformation had occurred.

Portions of the putatively transformed calli were then moved to Regeneration Medium I (MSO I) containing cefotaxime and hygromycin and kept in the dark room at room temperature for a week and were subsequently moved to the light for regeneration. The tiny shoots were separated and transferred to deep petri plates containing Regeneration Medium II (MSO II) to promote root growth and hygromycin and cefotaxime to maintain respectively selection of the transformants and kill any remaining *Agrobacterium*.

EXAMPLE 4

15 *Agrobacterium*-Mediated Transformation of Velvet Bentgrass With Superbinary Hybrid Vector pSB111SH

In this example, an *Agrobacterium* transformation system for velvet bentgrass, *Agrostis canina* L., is described, which utilizes the superbinary hybrid test vector described in Example 1.

Media: All media were formulated and prepared as described in Example 3.

25

Production of regenerable callus: Mature seeds of velvet bentgrass were surface-sterilized and plated on MMSG medium. The plates were kept in the dark at room temperature for 3-6 weeks. The proliferating calli were selected and transferred to new MMSG medium on a regular basis. Callus chosen for transformation was transferred to new MMSG medium prior to co-cultivation to promote active cell division. The nature of the callus (*i.e.*, friability, regenerability and active growth) is believed to play a key role in obtaining efficient transformation.

- 30 -

Preparation of *Agrobacterium tumefaciens*

suspension: *Agrobacterium tumefaciens* LBA4404, harboring vector pSB111SH, was streaked from a glycerol stock stored at -80°C and grown at 28°C on plates containing AB medium, supplemented with 10 µg/ml tetracycline and 50 µg/ml spectinomycin. After three to six days, the bacterial lawn was scraped from one (82 mm diameter) plate and suspended in 6 ml inoculation medium containing 200 µM acetosyringone. The bacterial suspension was left at 28°C in the dark with shaking overnight. In the morning acetosyringone was added to a final concentration of 400 µM and pluronic F-68 to 0.02%.

Co-cultivation: A sterile filter was placed on a co-cultivation plate containing acetosyringone (200 µM) and glucose (10 gm/l). Onto this filter was placed one large clump of friable callus which was then gently broken up and dispensed evenly over the filter. About 500 µl of *Agrobacterium* suspension was pipetted evenly onto the callus. The plates were sealed with parafilm and placed in the dark at room temperature for three days.

Selection and regeneration of transformants:

Subsequent to the three day co-cultivation, the co-cultivated calli were rinsed with 250 µg/ml cefotaxime solution to suppress bacterial growth, and the calli were placed on MMSG medium containing 200 µg/ml hygromycin and 250 µg/ml cefotaxime. The calli were kept in the dark at room temperature for 6-8 weeks and checked periodically for proliferation of the calli on hygromycin. The hygromycin-resistant calli were moved to new MMSG plates with hygromycin and kept in the dark at room temperature until well proliferated. Then a portion of the hygromycin-resistant callus was tested for GUS activity to ensure that transformation had occurred.

- 31 -

Portions of the putatively transformed calli were then moved to Regeneration Medium I (MSO I) containing cefotaxime and hygromycin and were kept in the dark room at room temperature. After one week, they were moved to the light for regeneration. The tiny shoots were separated and transferred to deep petri plates containing Regeneration Medium II (MSO II) to promote root growth and hygromycin and cefotaxime to maintain respectively selection pressure and kill any remaining *Agrobacterium* cells.

REFERENCES

- Aldemita, R.R. and T.K. Hodges (1996)
Agrobacterium tumefaciens-mediated transformation of
japonica and indica rice varieties. *Planta* 199:612-617.
- Belanger, F.C., C.L. Laramore, and P.R. Day
(1996) Turfgrass biotechnology. *Rutgers Turfgrass
Proceedings* 28:1-3.
- Cheng, M., J.E. Fry, S. Pang, H. Zhou, C.M.
Hironaka, D.R. Duncan, T.W. Conner and Y. Wan (1997)
Genetic transformation of wheat mediated by *Agrobacterium
tumefaciens*. *Plant Physiology* 115:971-980.
- Czernilofsky, A.P., R. Hain, L. Herrera-
Estrella, H. Lorz, E. Goyvaerls, B.J. Baker and J. Schell
(1986) Fate of selectable marker DNA integrated into the
genome of *Nicotiana tabacum*. *DNA* 5:101-113.
- Hiei, Y., S. Ohta, T. Komari and T. Kumashiro
(1994) Efficient transformation of rice (*Oryza sativa* L.)
mediated by *Agrobacterium* and sequence analysis of the
boundaries of the T-DNA. *Plant Journal* 6:271-282.
- Ishida, Y., H. Saito, S. Ohta, Y. Hiei, T.
Komari and T. Kumashiro (1996) High efficiency
transformation of maize (*Zea mays* L.) mediated by
Agrobacterium tumefaciens. *Nature Biotechnology* 14:745-

- 32 -

750.

Keller, T., H.G. Damude, D. Werner, P. Doerner, R.A. Dixon and C. Lamb (1998) A plant homolog of the neutrophil NADPH oxidase gp91^{phox} subunit gene encodes a plasma membrane protein with Ca²⁺ binding motifs. *Plant Cell* 10:255-266.

Knipple, D.C., C.L. Rosenfield, S.J. Miller, W. Liu, J. Tang, P.W.K. Ma and W.L. Roelofs (1998) Cloning and functional expression of a cDNA encoding a pheromone gland-specific acyl-CoA Δ^{11} -desaturase of the cabbage looper moth, *Trichoplusia ni*. *Proc. Natl. Acad. Sci. USA* 95:15287-15292.

Komari, T. (1990) Transformation of cultured cells of *Chenopodium quinoa* by binary vectors that carry a fragment of DNA for the virulence region of pTiB0542. *Plant Cell Rep.* 9:303-306.

Komari, T., Y. Hiei, Y. Saito, N. Murai, and T. Kumashiro (1996) Vectors carrying two separate T-DNAs for co-transformation of higher plants mediated by *Agrobacterium tumefaciens* and segregation of transformants free from selection markers. *Plant J.* 10: 165-174.

Lee, L. (1996) Turfgrass biotechnology. *Plant Science* 115:1-8.

Lodge, J., W.K. Kaniewski and N.E. Tumer (1993) Broad spectrum virus resistance in transgenic plants expressing pokeweed antiviral proteins. *Proc. Natl. Acad. Sci. USA* 90: 7089-7093.

Manuel de la Fuente, J., V. Ramires-Rodriguez, J.L. Cabrera-Ponce and L. Herrera-Estrella (1997) Aluminum tolerance in transgenic plants by alteration of citrate synthesis. *Science* 276:1566-1568.

May, G.D., R. Afza, H.S. Mason, A. Wiecko, F.J. Novak and C.J. Arntun (1995) Generation of transgenic banana (*Musa acuminata*) plants via *Agrobacterium*-mediated transformation. *Biotechnology* 13:486-492.

- 33 -

- Meesters, P.A.E.P., J. Springer and G. Eggink (1997) Cloning and expression of the Δ -9 fatty acid desaturase gene from *Cryptococcus curvatus* ATCC 20509 containing histidine boxes and a cytochrome b_5 domain. *Appl. Microbiol. Biotechnol.* 47:663-667.
- 5 Mittler, R., V. Shulaev and E. Lam (1995) Coordinated activation of programmed cell death and defense mechanisms in transgenic tobacco plants expressing a bacterial proton pump. *Plant Cell* 7: 29-42.
- 10 Saito, Y. T. Komari, C. Matsuta, Y. Hayashi, T. Kumashiro and Y. Takanami (1992) Cucumber mosaic virus-tolerant transgenic tomato plants expressing satellite RNA. *Theor. App. Genet.* 83: 679-683.
- 15 Stukey, J.E., V.M. Mc Donough and C.E. Martin (1990) The *ole1* gene of *Saccharomyces cerevisiae* encodes the Δ -9 fatty acid desaturase and can be functionally replaced by the rat stearyl-CoA desaturase gene. *J. Biol. Chem.* 265:20144-20149.
- 20 Tingay, S., D. McElroy, R. Kalla, S. Fieg, M. Wang, S. Thornton and R. Brettell (1997) *Agrobacterium tumefaciens*-mediated barley transformation. *Plant Journal* 11:1369-1376.
- 25 Wang, C., C.-K. Chin and A. Chen (1998) Expression of the yeast delta nine desaturase gene in tomato enhances its resistance to powdery mildew. *Physiological and Molecular Plant Pathology* 38:255-263.
- 30 Wu, G., B.J. Shortt, E.B. Lawrence, E.B. Levine, K.C. Fitzsimmons and D.M. Shah (1995) Disease resistance conferred by expression of a gene encoding H_2O_2 -generating glucose oxidase in transgenic potato plants. *Plant Cell* 7:1357-1368.
- Yuan, R. (1998) Biotech driving innovations in rice cultivation. *Genetic Engineering News*, Jan. 15 issue: 13-32.

- 34 -

Zoubenko, O., F. Uckun, Y. Hur, I. Chet and N. Tumer (1997) Plant resistance to fungal infection induced by nontoxic pokeweed antiviral protein mutants. *Nature Biotech.* 15: 992-996.

5

While certain of the preferred embodiments of the present invention have been described and specifically exemplified above, it is not intended that the invention be limited to such embodiments. Various
10 modifications may be made thereto without departing from the scope and spirit of the present invention, as set forth in the following claims.

- 35 -

What is claimed is:

1. A method of producing a transgenic turfgrass plant, comprising the steps of:

- 5 a) providing regenerable callus tissue from the turfgrass plant;
- b) inoculating the tissue with *Agrobacterium* carrying at least one vector for transformation, the vector comprising virulence genes
- 10 that confer strong infectivity to *Agrobacterium*, in which vector is inserted a heterologous DNA construct operably linked to a promoter from a monocotyledonous species, and a selectable marker gene conferring antibiotic resistance to transformed cells operably linked to a promoter from a
- 15 monocotyledonous species;
- c) culturing the inoculated tissue under conditions that enable the *Agrobacterium* vector to transform cells of the tissue;
- d) selectively culturing the inoculated
- 20 tissue on a selection medium comprising the antibiotic; and
- e) regenerating a transformed turfgrass plant from the selectively cultured tissue.

25 2. The method of claim 1, wherein the turfgrass is a species selected from the group consisting of creeping bentgrass, tall fescue, velvet bentgrass, perennial ryegrass, hard fescue, Chewings fescue, strong creeping fescue, colonial bentgrass and Kentucky

30 bluegrass.

3. The method of claim 1, wherein the *Agrobacterium* comprises a binary vector system and the virulence genes therein are obtained from a plasmid

35 within *Agrobacterium tumefaciens* strain 281.

- 36 -

4. The method of claim 3, wherein the binary vector system comprises plasmid pSB111SH.

5. The method of claim 1, wherein the promoter is selected from the group consisting of maize ubiquitin gene promoters, rice actin gene promoters, maize *Adh* 1 gene promoters, rice or maize tubulin (*Tub* A, B or C) gene promoters, and alfalfa *His* 3 gene promoters.

6. The method of claim 1, wherein the selectable marker gene confers hygromycin resistance on transformed tissue.

7. The method of claim 1, wherein the callus is obtained by culturing seeds of the turfgrass on a medium that promotes de-differentiation of plant tissue.

8. A transgenic turfgrass plant prepared by the method of claim 1.

9. A seed of the transgenic turfgrass plant of claim 8.

10. The transgenic turfgrass plant of claim 8, which comprises a transgene selected from the group consisting of:

- a) a gene encoding glucose oxidase;
- b) a gene encoding citrate synthase;
- c) genes encoding Δ -9 desaturase from *Saccharomyces cerevisiae* or *Cryptococcus curvatus*;
- d) a gene encoding Δ -11 desaturase;
- e) a gene encoding a plant homolog of the neutrophil NADPH oxidase;
- f) a gene encoding bacteriopsin from *Halobacterium halobium*; and

- 37 -

g) a gene encoding pokeweed antiviral protein.

11. A superbinary vector system for
5 *Agrobacterium*-mediated transformation of turfgrass, which comprises:

a) a virulence region from a Ti plasmid of an *A. tumefaciens* strain that confers to the strain as strong a virulence as that displayed by *A. tumefaciens*
10 strain 281;

b) a selectable marker gene operably linked to a promoter obtained from a gene of a monocotyledenous plant; and

c) a site for insertion of at least one
15 additional coding sequence, operably linked to a promoter obtained from a gene of a monocotyledenous plant, the promoter being the same as or different from the promoter operably linked to the selectable marker gene.

12. The vector system of claim 11, wherein the
20 virulence region is obtained from *Agrobacterium* strain 281.

13. The vector system of claim 11, wherein the promoter is selected from the group consisting of maize
25 ubiquitin gene promoters, rice actin gene promoters, maize *Adh* 1 gene promoters, rice or maize tubulin (*Tub* A, B or C) gene promoters, and alfalfa *His* 3 gene promoters.

14. The vector system of claim 11, wherein the
30 selectable marker gene confers hygromycin resistance on transformed cells.

15. The vector system of claim 11, wherein the site for insertion of the additional coding sequence
35 comprises a coding sequence of a reporter gene.

- 38 -

16. The vector system of claim 15, which comprises plasmid pSB111SH.

5 17. The vector system of claim 11, wherein the site for insertion of the additional coding sequence comprises a coding sequence of a gene selected from the group consisting of:

- 10 a) a gene encoding glucose oxidase;
b) a gene encoding citrate synthase;
c) genes encoding Δ -9 desaturase from *Saccharomyces cerevisiae* or *Cryptococcus curvatus*;
d) a gene encoding Δ -11 desaturase;
e) a gene encoding a plant homolog of the
15 neutrophil NADPH oxidase;
f) a gene encoding bacteriopsin from *Halobacterium halobium*; and
g) a gene encoding pokeweed antiviral
protein.

20 18. A turfgrass plant cell transformed with the vector system of claim 11.

25 19. The turfgrass plant cell of claim 11, wherein the cell is of a turfgrass selected from the group consisting of creeping bentgrass, tall fescue, velvet bentgrass, perennial ryegrass, hard fescue, Chewings fescue, strong creeping fescue, colonial bentgrass and Kentucky bluegrass.

30 20. A transgenic turfgrass plant regenerated from the transformed cell of claim 18.

35 21. A seed of the transgenic turfgrass plant of claim 20.

COMBINED DECLARATION AND POWER OF ATTORNEY

**(ORIGINAL, DESIGN, NATIONAL STAGE OF PCT, SUPPLEMENTAL, DIVISIONAL,
CONTINUATION, OR C-I-P)**

As a below named inventor, I hereby declare that:

TYPE OF DECLARATION

This declaration is for a national stage of PCT application.

INVENTORSHIP IDENTIFICATION

My residence, post office address and citizenship are as stated below, next to my name. I believe that I am an original, first and joint inventor of the subject matter that is claimed, and for which a patent is sought on the invention entitled:

TITLE OF INVENTION

Agrobacterium-Mediated Transformation of Turfgrass

SPECIFICATION IDENTIFICATION

The specification was filed on July 15, 1999, as International Application Number PCT/US99/16001.

ACKNOWLEDGMENT OF REVIEW OF PAPERS AND DUTY OF CANDOR

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information, which is material to patentability as defined in 37, Code of Federal Regulations, Section 1.56.

CLAIM FOR BENEFIT OF PRIOR U.S. PROVISIONAL APPLICATION(S)
(35 U.S.C. Section 119(e))

I hereby claim the benefit under Title 35, United States Code, Section 119(e) of any United States provisional application(s) listed below:

PROVISIONAL APPLICATION NUMBER

FILING DATE

60/093,163

July 17, 1998

POWER OF ATTORNEY

I hereby appoint the following practitioner(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

APPOINTED PRACTITIONER(S)

REGISTRATION NUMBER(S)

Janet E. Reed

36,252

Alfred W. Zaher

42,248

Bruce D. George

43,631

Teresa O. Bittenbender

P47,425

SEND CORRESPONDENCE TO

DIRECT TELEPHONE CALLS TO:

Janet E. Reed
215-972-8386

Janet E. Reed
Centre Square West
1500 Market Street, 38th Floor
Philadelphia, PA 19102-2186
US

DECLARATION

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

SIGNATURE(S)

100
Barbara A. Zilinskas

Inventor's signature Barbara A. Zilinskas

Date January 11, 2001

Country of Citizenship US

Residence Princeton, NJ 08540 NJ

Post Office Address 31 Washington Avenue, Princeton, NJ 08540 US

200
Lynne H. Pitcher

Inventor's signature Lynne H. Pitcher

Date 1/11/2001

Country of Citizenship US

Residence Highland Park, NJ 08904

Post Office Address 100 S. First Avenue, Highland Park, NJ 08904 US

200
Subha Lakkaraju

Inventor's signature Subha Lakkaraju

Date 1/11/2001

Country of Citizenship India

Residence New Brunswick, NJ US

Post Office Address 39 D Phelps Avenue, New Brunswick, NJ 08903 US

16 Thom Court, East Brunswick, NJ 08816 US